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SUMMARY STATEMENT
(Privileged Communication)

Release Date: 06/28/2008

Application Number: 1 R01 AI072021-01A2

Principal Investigator

HUSTON, CHRISTOPHER D MD

Applicant Organization: UNIVERSITY OF VERMONT & ST AGRIC COLLEGE

Review Group: ZRG1 IDM-R (02)
Center for Scientific Review Special Emphasis Panel
Eukaryotic Pathogens

Meeting Date: 06/19/2008 *RFA/PA:* PA07-070
Council: OCT 2008 *PCC:* M40 B
Requested Start: 12/01/2008

Project Title: Molecular Mechanism of Entamoeba histolytica phagocytosis

SRG Action: Priority Score: 118 Percentile: 1.1 #

Human Subjects: 10-No human subjects involved

Animal Subjects: 10-No live vertebrate animals involved for competing appl.

Project Year	Direct Costs Requested	Estimated Total Cost
1	200,000	301,000
2	200,000	301,000
3	200,000	301,000
4	200,000	301,000
5	200,000	301,000
<hr/> TOTAL	<hr/> 1,000,000	<hr/> 1,505,000

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

NEW INVESTIGATOR

1R01AI072021-01A2 HUSTON, CHRISTOPHER

NEW INVESTIGATOR

RESUME AND SUMMARY OF DISCUSSION: This outstanding and well-written application proposes to delineate mechanisms by which *Entamoeba histolytica* recognizes and induces phagocytosis of colonic bacteria and host cells. The knowledge of the events which initiate phagocytosis by invasive *E. histolytica* will be of high impact in understanding the pathogenesis of this parasite. In response to the prior critiques, the revised application is greatly improved and generates excitement by adding a new aim and additional experiments such as transcriptional gene silencing and development of dominant-negative mutant. There are numerous strengths and no weaknesses have been noted. Novel hypothesis, well-developed research plan, innovative techniques, outstanding investigator and collaborators, impressive publications and strong and convincing preliminary data which support the feasibility of the proposed studies garner high enthusiasm for this application. There is high confidence in the success of the proposed studies.

DESCRIPTION (provided by applicant): Amebiasis ranks second as a protozoan cause of death. *Entamoeba histolytica*, the etiologic agent, is an intestinal ameba that acquires nutrients by phagocytosis of colonic bacteria, and phagocytosis of host cells by *E. histolytica* is a prominent feature of invasive amebiasis. Despite its importance, little is known about the mechanism of *E. histolytica* phagocytosis. *Entamoeba histolytica* induces host cell apoptosis, resulting in host cell surface changes that engage unknown amebic phagocytosis receptors. In mammals, collectin family members (e.g., mannose binding lectin (MBL)) and the structurally related protein C1q bind to apoptotic cells, and initiate phagocytosis by interaction of their conserved collagenous "tails" with calreticulin. Calreticulin, which has no transmembrane domain, serves as a bridge between collectins and the macrophage receptor CD91. Preliminary data show that: 1) C1q, MBL, and collectin tails stimulate *E. histolytica* phagocytosis, 2) C1q and MBL compete for binding to the surface of *E. histolytica*, 3) calreticulin is present on the amebic surface and re-localizes to the phagocytic cup during interaction with apoptotic cells, 4) human C1q and the collectins MBL and SP-A compete for binding to immobilized amebic calreticulin, and 5) specific *E. histolytica* surface proteins interact with calreticulin. Therefore, host collectins bound to apoptotic cells and bacteria may stimulate *E. histolytica* phagocytosis by interaction of their collagenous tail domain with an amebic receptor. Calreticulin is one logical candidate, and, if calreticulin participates in amebic phagocytosis, it is hypothesized to do so by bridging between collectins and a calreticulin receptor on the amebic surface. The specific aims test these hypotheses. In aim 1, purified collectins, C1q, and collagenous collectin "tails" will be used to test if *E. histolytica* has a receptor for the collectin tail that mediates engulfment of apoptotic cells. Except for C1q, the collectins also opsonize bacteria in mucosal secretions. Therefore, studies will be performed to test if *E. histolytica* engulfs apoptotic cells and bacteria via the same mechanism. In aim 2, RNA-mediated interference and an alternative gene silencing method will be used to determine if calreticulin participates in *E. histolytica* phagocytosis. Recombinant calreticulin will be used for binding studies to determine if *E. histolytica* has a calreticulin receptor. In aim 3, either a collectin or a calreticulin receptor will be identified. The decision of which to pursue will depend on the results from aims 1 and 2. In either case, cross-linking methods and complementary affinity-based methods will be combined with mass spectrometry to identify interacting amebic surface proteins. Successful completion of the proposed studies will provide a molecular understanding of *E. histolytica* phagocytosis, and will substantially augment our knowledge of how *E. histolytica* interacts with the host and with colonic bacteria. Furthermore, new insights into the pathogenesis of amebiasis that result from these studies may suggest novel methods for its treatment and prevention. **PUBLIC HEALTH RELEVANCE:** *Entamoeba histolytica*, a single-celled intestinal parasite that causes invasive amebiasis (a disease characterized by bloody diarrhea and liver abscesses), engulfs killed cells during invasion through host tissues. The goal of this project is to determine the molecular mechanisms underlying *E. histolytica*'s ability to recognize and engulf killed cells. This will provide novel insights into how *E. histolytica* causes invasive infections, possibly suggesting new methods to treat or prevent amebiasis.

CRITIQUE 1:

Significance: *E. histolytica* is a major cause of morbidity and mortality world-wide, particularly in developing countries. It is also a Class B Agent because of the potential for water-borne spread. The parasite obtains nutrients by phagocytosis of bacteria and apoptotic host cells, but the mechanisms are poorly understood. *E. histolytica* induces apoptosis of host cells, which are more efficiently phagocytosed than live cells. Although the signaling and vesicle trafficking steps of phagocytosis have begun to be defined, the actual mechanisms triggering phagocytosis have not. Further understanding of the events triggering phagocytosis by invasive *E. histolytica* will be a very important step in understanding its pathogenesis.

Approach: This outstanding revised proposal is based on significant new preliminary data to identify the key molecules in triggering amebic phagocytosis. One potential candidate is the collectin family, which bind to apoptotic cells and bacteria. Calreticulin, a soluble ER chaperone, also binds the collagenous collectin tail and is surface associated in many cell lines. A calreticulin homologue has been found in the *E. histolytica* genome project and in proteomic studies of amebic phagosomes. There is no homologue for the usual calreticulin binding protein, CD91, in *E. histolytica*, leading to the key hypothesis of this application that host collectins trigger amebic phagocytosis by binding to calreticulin. This is supported by preliminary data that apoptotic lymphocytes or latex beads were more efficiently opsonized when coated with C1q (subunit of complement component 1 which has the same collagenous tail), mannose binding lectin, or human surfactant A tails.

Specific Aim 1 is directed at identifying a phagocytosis receptor for the common collagenous tail. The investigator has all the key reagents to test multiple potential targets, C1q tails, intact and biotinylated C1q, MBL, and SP-A tails. The potential problems in dissecting the effect on what may be one of multiple systems of uptake are appropriately discussed. In the third part of the Aim, the potential binding through sugars will be assessed by treating C1q on beads with N-glycanase. If consistent deglycosylation was not possible, one alternative approach was to express the target proteins in *E. coli*, but it will be very difficult to reconstitute such as large protein as C1q, which has 18 peptide chains. The fourth part of Aim 1 will assess the role of collectins in phagocytosing both apoptotic cells and GFP-expressing *E. coli*. The major problem that there actually is no receptor was brought up in past reviews. They now provide other alternative experimental approaches such as identifying alternative lectins and considering other ligands on the surface of apoptotic cells.

Aim 2 focuses on the interaction of calreticulin with a transmembrane signaling partner. This Aim is supported by preliminary data showing the correct localization of epitope-tagged calreticulin, which results in increased phagocytosis and new anti-calreticulin antibodies that will be key for specificity studies. The first approach will be to silence calreticulin by using a system expressing short hairpin RNAs developed by their collaborator, Dr. Petri. This system has been used effectively to silence a Gal/GalNac subunit and a transmembrane kinase, which is particularly impressive because of the apparent specificity with >90 transmembrane kinases. The second approach would be to use a transcriptional gene silencing method, whose mechanism has not been clearly delineated. The exploitation of a dominant-negative mutant, which is proposed as an alternative later, would be more straight-forward, if effective. Biotinylated recombinant calreticulin would be used to dissect the role of surface calreticulin, which could be problematic because it is not known how calreticulin associates with the membrane, but their preliminary data suggests it is bioactive. The new antibodies will be used in blocking experiments and the epitopes mapped.

The new third Aim is will identify either the collectin or calreticulin receptor by using a cross-linker to transfer a biotin label from C1q tails or recombinant calreticulin ("the bait protein") to the "binding protein" or biotinylation of surface proteins in the calreticulin overexpressing amebae and immunoprecipitation A third approach would use C1q tails to co-immunoprecipitate biotinylated surface proteins. They identify reasonable ways to prioritize identified proteins as many will be likely be co-

purified. To confirm their findings, they will examine the *in vitro* interactions of the recombinant proteins, including the extracellular domain of the putative receptor. This may be problematic as the interaction of membrane associated proteins may be difficult to duplicate with soluble proteins so the alternative approach expressing the proteins in CHO cells will likely have to be used. Finally, the identified candidate proteins will be down-regulated with RNAi using techniques optimized with calreticulin.

Innovation: The hypothesis that calreticulin is likely a key phagocytosis receptor in *E. histolytica* is novel and coupled with the adaptation of the newest techniques such as RNAi, genetic silencing, antibodies developed from phage displays, and bait proteins with cross-linking to identify receptors are innovative.

Investigators: Dr. Christopher Huston, the investigator, is an Assistant Professor at the University of Vermont after spending 5 years as an Infectious Disease and postdoctoral fellow in the laboratory of Dr. Bill Petri at University of Virginia. He was first author on some of the key papers leading to the proposed studies and is well-qualified to carry them out. His key collaborators, including Dr. Bill Petri, Dr. Tomoyoshi Nozaki, and Dr. Gary Ward are all internationally recognized leaders in their fields of amebic pathogenesis, phagocytosis, and protozoan virulence.

Environment: The University of Vermont has an experienced group of parasitic investigators who will provide a rich scientific environment.

Response to Previous Review: This is an outstanding revised submission in which the investigator has made excellent progress in addressing previous critiques. The major concern focused on lack of scientific options if calreticulin was not the key phagocytosis receptor for *E. histolytica*, which could be confirmed by RNAi studies. They have now added additional options, including transcriptional gene silencing and developing a dominant-negative mutant. Most importantly, there is a new Aim 3, in which alternative approaches are outlined to identify receptors other than calreticulin. Other critiques have also been addressed, including additional experiments to test specificity of the system for apoptotic cells as well as bacteria and more detailed experiments to optimize the reagents developed to test the specificity for the receptor for protein vs. sugars. For Aim 2, a focus on anti-calreticulin antibodies to inhibit phagocytosis has been removed. An impressive amount of new preliminary data also supports the feasibility of the proposed studies. In response to a comment about modest productivity, the investigator includes four additional manuscripts, which are published or in press.

Overall Evaluation: This is an outstanding, well-written application, which addresses the molecular mechanisms of phagocytosis in *Entamoeba histolytica*. The investigator has responded well to previous critiques and has a focused proposal that is supported by significant preliminary data and new publications. The overall hypothesis of the involvement of key phagocytosis receptors in *E. histolytica* as well as the multiple approaches used to define them; including genetic manipulation, active recombinant proteins, and flow-based quantitative assays are innovative. The potential pitfalls of RNAi as well as alternative approaches should alternative hypotheses need to be considered are well-addressed.

Biohazards: The investigator has extensive experience with a BSL-2 pathogen.

Budget: Appropriate

CRITIQUE 2:

Significance: *Entamoeba histolytica* (Eh), which causes amebic dysentery and amebic liver abscess, is a major cause of parasite-related morbidity and mortality worldwide. Although phagocytosis of host cells by the parasite is a hallmark of invasive disease caused by Eh, little is known about the molecular mechanisms underlying this process. The focus of this application is to investigate the role of host collectins and calreticulin or other parasite receptors in this process. The proposed studies will lead to a

better understanding of the pathogenesis of invasive amebiasis and may identify new targets for prevention.

Approach: The hypothesis of the revised application is that host collectins bound to apoptotic cells or bacteria trigger phagocytosis via a parasite collectin receptor, the leading candidate for which is calreticulin. The investigator presents extensive and convincing preliminary data, some of which is now published, in support of this interesting and novel hypothesis. He has shown that opsonization of apoptotic cells or beads with C1q, collectins, or their purified collagenous tails stimulates Eh phagocytosis, that calreticulin is present on the amebic surface and in phagosomes and binds directly to apoptotic cells, that C1q binds to parasite calreticulin and that Eh surface proteins co-immunoprecipitate with calreticulin. He has developed assays to study binding and phagocytosis and generated reagents such as antibodies and recombinant proteins to enable him to test this hypothesis.

There are 3 specific aims in the current application. In the first aim the investigator proposes to determine whether Eh has a phagocytosis receptor specific for the collagenous collectin tail. Aim 1.1 addresses stimulation of phagocytosis by specific binding of C1q tails. As the investigator and a previous reviewer note, much of this has been completed already. Aim 1.2 will focus on binding specificity; Aim 1.3 will determine whether binding is carbohydrate-mediated and Aim 1.4 will focus on collectin-dependent phagocytosis of apoptotic cells and colonic bacteria. The experiments proposed in this aim are based on a clear rationale and are straightforward and well-described. Appropriate controls are included, possible pitfalls and alternative explanations and approaches have been carefully considered. One alternative explanation proposed if it is found that Eh does not have a receptor for the collection tail is that an as yet unknown parasite lectin mediates uptake of C1q-coated particles. Although this seems unlikely given that lectins in Eh have been extensively investigated previously, this could be investigated using a panel of sugars (other than Gal or GalNAc) to inhibit the interaction.

The second aim is directed at investigating whether calreticulin is the Eh phagocytosis receptor that stimulates phagocytosis by interaction with a transmembrane signaling partner. Two gene silencing approaches are proposed to determine this in Aim 2.1. The first is RNAi using a method developed by Dr. Petri. The second, proposed in response to a concern from the previous review is a transcriptional silencing approach developed by Dr. David Mirelman, who has provided the necessary plasmids. These approaches are appropriate and feasible and the investigator has experience with at least one of them. Important experiments to ensure specificity of the knockdown for phagocytosis are included. Generation of a dominant negative mutant is proposed as an alternative. In Aim 2.2 the investigator will determine whether surface calreticulin is involved using soluble recombinant calreticulin and in Aim 2.3 he proposes to determine if amebic surface calreticulin is a collectin receptor, and if it binds directly to target cells and bacteria. Use of the calreticulin-deficient parasites generated in 2.1 is proposed as an alternative, but could be included as part of the initial approach. Aim 2.4 is directed at mapping the calreticulin domains involved. Although experiments in these latter subaims are well-thought out and well-described, their relevance is contingent upon evidence from Aim 2.1 that calreticulin is indeed an Eh phagocytosis receptor.

Aim 3 has been added in response to a major concern of the previous review that RNAi may reveal that phagocytosis is not affected by knockdown of calreticulin. Although the preliminary studies strongly implicate calreticulin as the phagocytosis receptor, if experiments in Aim 2 indicate that this is not the case, the possibility of an alternative collectin receptor will be investigated. The methodology proposed in aim 3.1 for identification of either receptor is similar and involves 3 cross-linking or immunoaffinity and proteomics-based approaches that will be done in collaboration with Dr. Gary Ward, who has expertise in these techniques. The advantages and disadvantages of each method have been carefully considered, appropriate specificity controls included and a scheme for prioritization of candidate proteins designed. Aim 3.2 will confirm the interaction of the candidate protein with collection or calreticulin using *E. coli*-expressed recombinants and in Aim 3.3 the investigator will confirm the function of the candidate receptor using gene-silencing approaches as in Aim 2.

Innovation: The proposed approaches are not particularly innovative. However, the innovation of this application lies in the novel hypothesis proposed to explain the mechanisms underlying amebic phagocytosis.

Investigators: Dr. Huston, a talented new investigator, is Assistant Professor in the Department of Medicine at the University Of Vermont College Of Medicine since 2003. He received excellent post-doctoral training in the laboratory of Dr. Bill Petri at the University Of Virginia School Of Medicine. He has recently been very productive and has 4 relevant publications in 2008, including 3 as senior author. He is extremely well-qualified to conduct the proposed studies. In addition, Dr. Huston has recruited an impressive team of outstanding investigators as collaborators.

Environment: The environment at the University Of Vermont School Of Medicine is outstanding.

Response to Previous Review: The investigator has been highly responsive to the concerns and suggestions of the previous review. He has added a 3rd aim to address the possibility that calreticulin may not be the collectin receptor. In addition he has proposed alternative genetic approaches to RNAi. He has published 4 relevant peer-reviewed papers since the previous application. He has also included additional preliminary data.

Overall Evaluation: This is an outstanding application from a new investigator which addresses the mechanisms underlying *E. histolytica* phagocytosis of host cells and colonic bacteria. The strengths of the application include the significance and innovation of the project, the talented and now productive investigator and the outstanding team of collaborators. The research plan is well-planned and written and based on extensive and convincing preliminary data. The investigator has been highly responsive to the prior review.

Biohazards: *E. histolytica* is a BSL-2 pathogen which the investigator has experience with. No concerns.

Budget: In response to the previous review, in the A2 application the budget has been reduced to 8 modules, which is appropriate.

CRITIQUE 3:

Significance: Intestinal amoebiasis is a significant cause of global morbidity mortality. In many resource poor areas of the world, the infection may be almost universal (over the course of an individual's life), and intestinal amebiasis can lead to dysentery, colitis, intestinal perforation and metastatic seeding, especially resulting in hepatic abscesses and collections. An improved understanding of *E. histolytica* phagocytosis, a critical component of virulence in humans, would be highly significant.

Approach: This application proposes three specific aims. In aim one they propose to assess the hypothesis that *E. histolytica* has a phagocytosis receptor specific for collectins. In aim two, the investigators propose to test the hypothesis that cell surface calreticulin is an *E. histolytica* phagocytosis receptor that stimulates phagocytosis by interaction with a transmembrane signaling partner. In aim three, the investigators propose to identify the *E. histolytica* collectin or calreticulin receptor based on the results of specific aims one and two. The investigators have the reagents in hand, show preliminary results that they are capable of carrying out the proposed experiments, and have established a collaborative team with expertise both for providing reagents and for experimental assistance, and address possible pitfalls and alternative approaches.

Innovation: The use of shRNA interference studies and cross linking experiments as applied to the field of *E. histolytica* phagocytosis have innovative features.

Investigators: The team is outstanding.

Environment: Excellent

Response to a Previous Review: The investigator has carefully considered the two previous thoughtful reviews by the review panel and has appropriately and thoughtfully modified the project and the proposal, markedly strengthening the proposal.

Overall Evaluation: The strengths of the application include the significance of the question to be addressed, the preliminary data, the strength of the research team, and the experimental design. Identification of possible pitfalls and alternative approaches suggest that the investigators will be able to appropriately move the project forward, resulting in a significant advancement in our understanding of phagocytosis by *E. histolytica*, a critical part of virulence during human disease. Overall this is an outstanding proposal.

Biohazards: Appropriate

Budget: Appropriate.

CRITIQUE 4:

Overall Evaluation: The strengths of this application are a wealth of strong preliminary data and a well reasoned, thoughtfully written research plan. The investigator is clearly well qualified to carry out the proposed research which should provide new insight into the mechanism into *E. histolytica* phagocytosis.

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

Ad hoc or special section application percentiled against "Total CSR" base.

NOTICE: The NIH has modified its policy regarding the receipt of amended applications. Detailed information can be found by accessing the following URL address:
<http://grants.nih.gov/grants/policy/amendedapps.htm>

NIH announced implementation of Modular Research Grants in the December 18, 1998 issue of the NIH Guide to Grants and Contracts. The main feature of this concept is that grant applications (R01, R03, R21, R15) will request direct costs in \$25,000 modules, without budget detail for individual categories. Further information can be obtained from the Modular Grants Web site at <http://grants.nih.gov/grants/funding/modular/modular.htm>

MEETING ROSTER

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Eukaryotic Pathogens
ZRG1 IDM-R (02) M
June 19, 2008**

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Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.